Affinity membranes have also been suggested for use in extracorporeal circuits, for the removal of toxic substances such as certain metabolites or antibodies from blood. For example, exogenous human serum amyloid P component, a substance associated with Alzheimer's disease, has been removed from whole rat blood in an extracorporeal circulation system. This model system used a polyclonal antibody coupled to cellulose flat-sheet membranes. The biocompatibility of the membrane was also demonstrated. A similar application is the removal of autoantibodies from human plasma, using membrane-bound affinity ligands in extracorporeal circuits.

Apart from preparative applications, small cartridges with membrane discs or continuous membrane rods should be useful for analytical-scale separations and affinity solid-phase extraction, for example for immunoextraction.

Conclusions

Affinity membrane separation techniques combine the specificity of affinity adsorption with the unique hydrodynamic characteristics of porous membranes. They provide low pressure separation systems which are easy to scale up and ideal for the processing of large volumes of potentially viscous feed solutions (e.g. microbial broth, bacterial cell extract, conditioned media) often involved in the production of recombinant proteins. The additional microfiltration effect of membranes allows for the processing even of unclarified, particle-containing feed solutions. The high performance of this separation technique is due to the presence of through-pores and the absence of diffusional limitations; mass transfer is mainly governed by forced convection. Affinity membranes are used in applications such as purification of biomolecules, final product polishing, removal of unwanted substances from patients' blood in extracorporeal circuits, but also for smaller scale analytical separations. Biological affinity ligands and biomimetic or pseudobiospecific ligands are currently employed, as well as different membrane configurations such as flat sheets, hollow fibres or continuous rods. The technology is now in the process of being adapted more and more for large scale industrial separation and purification.

See also: **I/Affinity Separation. Membrane Separations. II/Affinity Separation:** Dye Ligands; Immunoaffinity Chromatography; Imprint Polymers; Rational Design, Synthesis and Evaluation: Affinity Ligands; **Chromatography: Liquid:** Large-Scale Liquid Chromatography. **Membrane Separations:** Filtration. **III/Immunoaffinity Extraction. Appendix 1/Essential Guides for Isolation/Purification of Enzymes and Properties. Essential Guides for Isolation/Purification of Immunoglobulins. Appendix 2/Essential Guides to Method Development in Affinity Chromatography.**

Further Reading

Brandt S, Goffe RA, Kessler SB, O'Connor JL and Zale SE (1988) Membrane-based affinity technology for commericial scale purifications. *Bio/Technology* 6: 779.

Charcosset C (1998) Purification of proteins by membrane chromatography. *Journal of Chemical Technology and Biotechnology* 71: 95.

Klein E (ed.) (1991) *Affinity Membranes: Their Chemistry and Performance in Adsorptive Separation Processes*. New York: John Wiley.

Roper DK and Lightfoot EN (1995) Separation of biomolecules using adsorptive membranes. *Journal of Chromatography* 702: 3.

Suen S-J and Etzel MR (1992) A mathematical model of affinity membrane bioseparations. *Chemical Engineering Science* 47: 1355.

Thömmes J and Kula MR (1995) Membrane chromatography – an integrative concept in the downstream processing of proteins. *Biotechnology Progress* 11: 357.

DropBooks

Affinity Partitioning in Aqueous Two-Phase Systems

G. Johansson, Center for Chemistry and Chemical Engineering, Lund University, Lund, Sweden

Copyright © 2000 Academic Press

Aqueous Two-phase Systems in General

The division of water into non-miscible liquid layers (phases) by addition of two polymers has led to the remarkable possibility of being able to partition proteins and other cell components between phases of nearly the same hydrophilicity. Proteins can be separated by partitioning if they have unequal distribution between the phases, i.e. when their partition coefficients, K (the concentration in top phase divided by the concentration in bottom phase), differ. Usually the difference in the K value of many proteins is not very large and then repeated extractions have to be carried out to get a reasonable

purification. If, however, the protein of interest (the target protein) has a very high K value and is mainly in the upper phase and all the contaminating proteins have very low K values so that they are in the bottom phase, an effective and selective extraction can be obtained in a single or a few partitioning steps. This type of partitioning has been made possible by using affinity ligands restricted to the upper phase.

The composition of the phases when two polymers like dextran and polyethylene glycol (PEG) are dissolved together in water depends on the amount of the polymers and their molecular weights. The concentration of the polymers in two phases of a given system can be found in the phase diagram for the temperature being used. A typical phase diagram is shown in **Figure 1**.

The line that connects the points in the diagram representing the compositions of the top and bottom phases of a system is called the tie-line. Each system with a total composition (percentage of each polymer) belonging to the same tie-line will have the same phase compositions. The smaller the tie-line, the more similar are the two phases in their composition. The greatest difference in composition of the top and bottom phases is therefore obtained by using high polymer concentrations.

The partitioning of proteins and also of membranes and particles depends on the polymer concentration of the system. The K value of a protein will be the same for all systems belonging to the same tie-line.

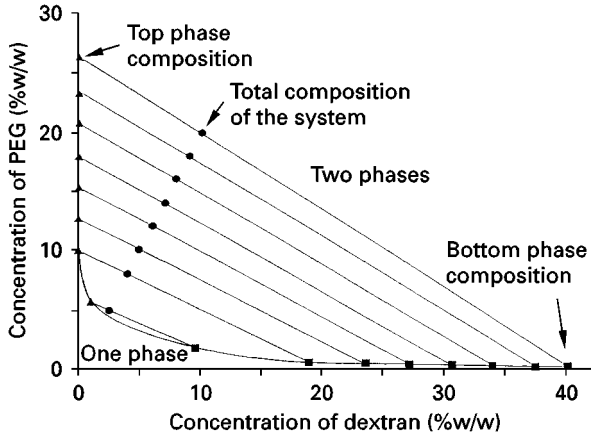


Figure 1 Phase diagram for the system dextran 500 (500 000 Da), PEG 8000 (8000 Da), and water at 23°C. Polymer compositions above the curved line (bimodal curve) give two liquid phases. All two-phase systems with their total composition on the same straight line (tie-line) have the same composition of top phase (▲) and bottom phase (■). The systems differ in phase volume ratio depending on their position on the tie-line. The indicated total compositions (●) give systems with more top phase (three to five times) than bottom phase.

The partition coefficient will, in most cases, decrease with the length of the tie-line, i.e. by using higher concentrations of the two polymers the material will accumulate more in the lower phase. Another way to affect the partitioning of proteins is by addition of salts to the system. Their effect depends on the type of cation and anion introduced with the salt. Negatively charged proteins show increasing K values when the cation is changed in the series:

$$K^+ < Na^+ < NH_4^+ < Li^+ < (C_4H_9)_4N^+$$

For the anion the partition coefficient increases in the following order:

$$ClO_4^- < SCN^- < I^- < Br^- < Cl^- < CH_3CO_2^- < F^- < H_2PO_4^- < HPO_4^{2-}$$

The highest K value of negatively charged proteins will then be obtained with the salt tetrabutylammonium hydrogenphosphate and the lowest K value with potassium perchlorate. Proteins with zero net charge (at their isoelectric points) are not affected by salts while positively charged proteins behave in an opposite manner to the negatively charged ones. For a number of proteins the log K values are nearly a linear function of their net charge (**Figure 2**).

Affinity Partitioning

The principle of affinity partitioning is to localize an affinity ligand in one phase to make it attract ligand-binding proteins. Since the phase-forming polymers are in each phase, either one can be used as ligand carrier. The standard system for affinity partitioning has been the one composed of dextran, PEG and water. Dextran is then used for localizing the ligand in the bottom phase while PEG can be used to concentrate the ligand to the top phase. PEG has often been chosen as ligand carrier because bulk proteins can be effectively partitioned into the dextran-rich lower phase by using high concentrations of polymers and a suitable salt. Thus, the target protein is extracted towards the upper phase leaving contaminating proteins in the bottom phase. PEG has two reactive groups (the terminal hydroxyl groups) which can be used as points of ligand attachment. In many cases only one ligand molecule is attached per PEG molecule. If the ligand is a large molecule (e.g. an antibody protein) several PEG chains may be attached to the one ligand molecule. Normally, only a fraction (1–10%) of the PEG in the two-phase system has to carry the ligand to reach maximal extraction efficiency. The more extreme the partitioning of a ligand–polymer is toward a phase the more effective it will be in extracting a ligand-binding protein into this phase. The partitioning of

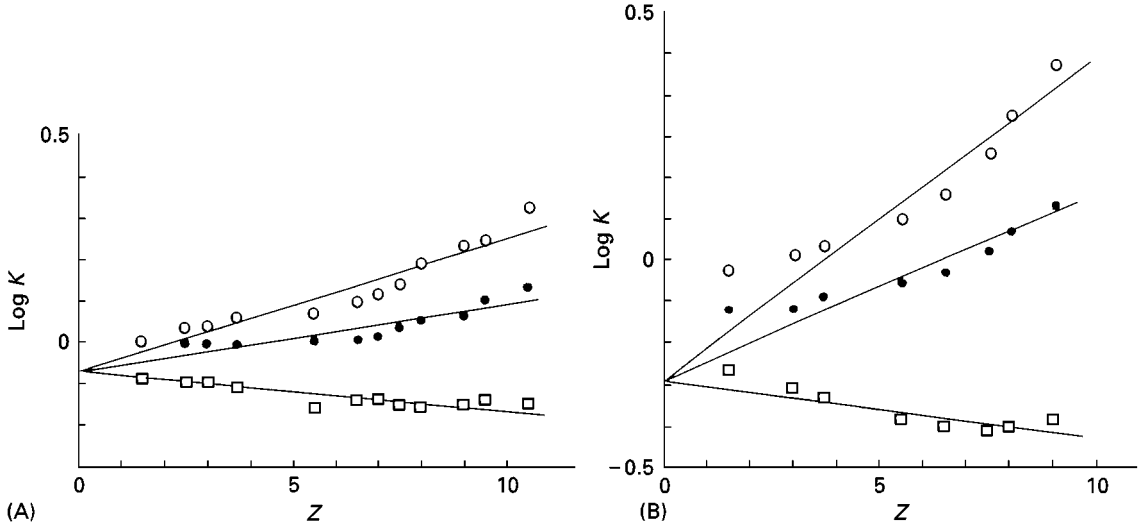


Figure 2 Log K of the protein ribonuclease-A as function of its net charge, Z, in a two-phase systems containing KSCN (○, 100 mM), KCl (●, 100 mM), or K_2SO_4 (□, 50 mM). System compositions: (A) 6.2%w/w dextran 500 and 4.4%w/w PEG 8000; (B) 9.8%w/w dextran 500 and 7.0%w/w PEG 8000. Protein concentration, 2 g L^{-1}. Temperature, 20°C. (Reprinted from Johansson G (1984) *Molecular Cell Biochemistry* 4: 169–180, with permission from Elsevier Science.)

the ligand–polymer should be in the same range as the non-derivatized polymer but it may, in some cases, be more extreme. The higher the polymer concentrations are in the system, i.e. the longer the tie-line of the system, the more extreme is the partitioning of PEG to the top phase and dextran to the bottom phase. This can be expressed by the partition coefficients of the two polymers:

$$K_{\mathrm{PEG}} = \frac{c_{\mathrm{PEG,top}}}{c_{\mathrm{PEG,bottom}}} \quad \text{and} \quad K_{\mathrm{dextran}} = \frac{c_{\mathrm{dextran,top}}}{c_{\mathrm{dextran,bottom}}}$$

where c is the respective polymer concentration in top or bottom phase. **Table 1** shows the K_{PEG} and K_{dextran} values for systems containing PEG 8000 and dextran 500. Dextran has a more extreme value of K than PEG, i.e. $K_{\mathrm{PEG}} < 1/K_{\mathrm{dextran}}$. Dextran should therefore, in principle, be a better ligand carrier than PEG. The concentration ratio for dextran is roughly the square of the ratio for PEG in the same system.

A Simple Theory for Affinity Partitioning

A basic theory for affinity partitioning was elaborated by Flanagan and Barondes in 1975. They analysed the combined binding and partition equilibria taking place in and between the two phases, respectively (**Figure 3**).

In this scheme the ligand–PEG(L), the free protein (P) and the two complexes (PL and PL_2) have each their own partition coefficient (K_L, K_P, K_{PL} and K_{PL_2}). Furthermore, in both phases association between protein and ligand–PEG takes place which can be described by the association constants:

$$K_1 = [\mathrm{PL}]/([\mathrm{P}][\mathrm{L}]) \quad \text{and} \quad K_2 = [\mathrm{PL_2}]/([\mathrm{PL}][\mathrm{L}])$$

one set for each phase.

A total association constant for the equilibrium:

$$\mathrm{P} + 2\mathrm{L} = \mathrm{PL_2}$$

can also be used: $K_{\mathrm{tot}} = K_1 K_2$.

Table 1 Partition coefficients of PEG (K_{PEG}) and dextran (K_{dextran}) and their logarithmic values (log) at various tie-line lengths of the system in Figure 1

Tie-line length (polymer concentration scale)	K_{PEG}	K_{dextran}	*log* K_{PEG}	*log* K_{dextran}
8.0	1.9	0.25	0.28	−0.60
14.2	6.7	0.023	0.83	−1.64
17.4	12	0.0088	1.08	−2.06
25.6	35	0.0022	1.54	−2.66
31	46	0.0004	1.66	−3.4
35	61	0.0001	1.79	−4.0

DropBooks

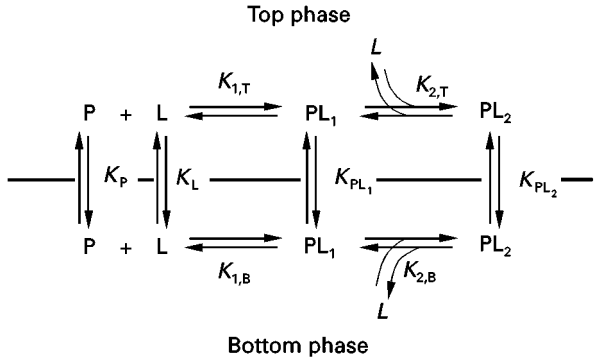


Figure 3 Scheme for affinity partitioning of a protein (P) with two binding sites for a ligand attached to PEG (L). The complexes between protein and ligand–PEG are PL and PL_2, respectively.

The association constants, K_{tot}, K_1 and K_2 may differ between the two phases. According to Flanagan and Barondes, the measured log K value of a protein, log $K_{protein}$, will, theoretically, give rise to a saturation curve when plotted versus the concentration of polymer-bound ligand in the system (compare **Figure 4**).

The log $K_{protein}$ value reaches a plateau when the concentration of L–PEG is so high that practically all the protein is present as the fully saturated complex PL_2. The protein molecule is then surrounded by two PEG chains and outwardly shows a PEG atmosphere.

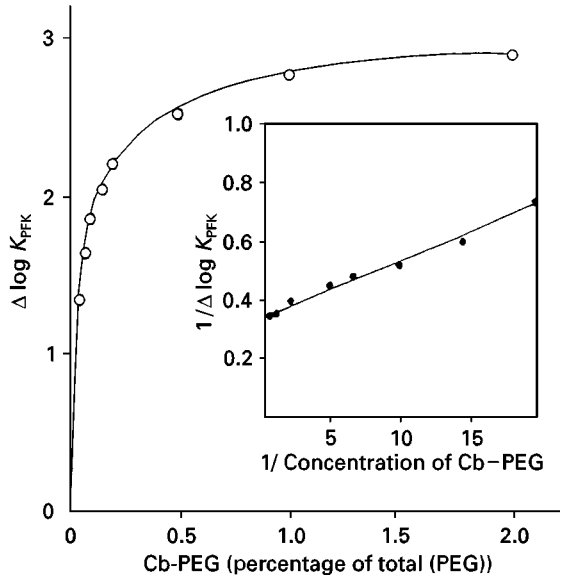


Figure 4 Increase in the logarithmic partition coefficient of phosphofructokinase (PFK) from bakers' yeast as function of the concentration of Cibacron blue F3G-A PEG (Cb-PEG). System composition: 7%w/w dextran 500, 5%w/w PEG 8000 including Cb–PEG, 50 mM sodium phosphate buffer pH 7.0, 0.5 mM EDTA, 5 mM 2-mercaptoethanol and 4 nkat g^{-1} enzyme. Temperature, 0°C. The inverse plot inserted is used to determine the Δ log K_{max}.

The maximum partition coefficient of protein, $\hat{K}_{protein}$ ($= K_{PL_2}$), is related to K_P, K_L and the K values via the following equations:

$$\hat{K}_{protein} = K_P K_L^2 \frac{K_{1,T} K_{2,T}}{K_{1,B} K_{2,B}}$$

or:

$$\hat{K}_{protein} = K_P K_L^2 \frac{K_{tot,T}}{K_{tot,B}}$$

The maximum increase in the logarithmic partition coefficient, $\Delta \log K_{max}$, is consequently given by:

$$\Delta \log K_{max} = \log \frac{\hat{K}_{protein}}{K_P}$$

$$= 2 \log K_L + \log K_{tot,T} - \log K_{tot,B}$$

If $K_{tot,T} = K_{tot,B}$ then $\Delta \log K_{max} = 2 \log K_L$.

From the values in Table 1 it may therefore be assumed that for proteins with two binding sites Δ log K_{max} can be as high as 3.57 (an increase of 3700 times in K) when PEG is used as ligand carrier with $K_L = 61$. If dextran is used as carrier, in the same system, the $\Delta \log K_{max}$ should theoretically be around -8 corresponding to a one hundred million times increase in the affinity of the protein for the lower phase if K_L is 0.0001. A higher number of binding sites (n) should then give strongly increasing Δ log K_{max} values with $\Delta \log K_{max} = n \log K_L$. However, the affinity extraction effect may be reduced by a reduction of individual binding strengths.

Experimental Results

The extraction curves of a protein, here exemplified with phosphofructokinase (PFK) from baker's yeast, using Cibacron Blue F3G-A PEG, closely follows the predicted behaviour (Figure 4). The inverse plot makes it possible to estimate the value of $\Delta \log K_{max}$.

The dependence of $\Delta \log K_{max}$ of PFK on the polymer concentration is shown in **Figure 5**. Increasing concentration of polymers corresponds to longer tie-line length (and greater K_L value) and this makes the affinity partitioning, measured as $\Delta \log K_{max}$, more efficient.

In addition to the concentration of polymers and ligand–PEG the actual $K_{protein}$ obtained also depends on pH value, the salt added to the system and the temperature. Two salts which have little or no effect on the affinity partitioning are phosphates and acetates in concentrations up to 50 mM. In the case of PEG the $\Delta \log K_{max}$ is reduced with increasing temperature.

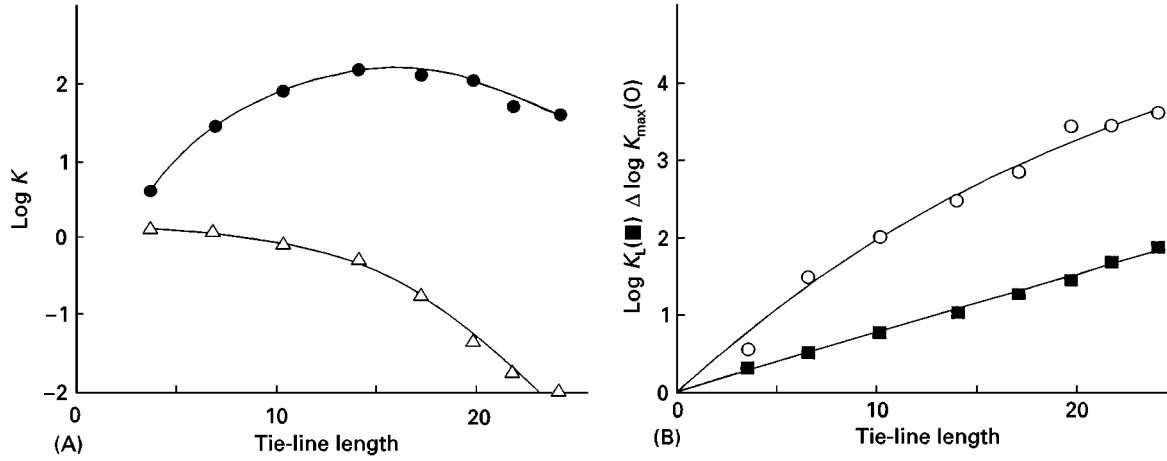


Figure 5 (A) Log K of phosphofructokinase from bakers' yeast as function of the tie-line length, expressed in the polymer concentration scale, in systems with an excess of Cibacron blue F3G-A PEG (Cb-PEG) (●), 3% of total PEG; or without Cb–PEG (Δ). (B) Δ log K_{max} (○) and log K_L (■) as function of the tie-line length. System composition: dextran 500 and PEG 8000 (including Cb–PEG) in weight ratio 1.5 : 1, 50 mM sodium phosphate buffer pH 7.0, 0.5 mM EDTA, 5 mM 2-mercaptoethanol, and 4 nkat g^{-1} enzyme. Temperature, 0°C.

The detachment of ligand from the enzyme can be achieved either by using a high concentration of salt or by the addition of an excess of free ligand. For PFK the addition of adenosine triphosphase (ATP) to the system containing ligand–PEG strongly reduces the partition coefficient of the enzyme (**Figure 6**).

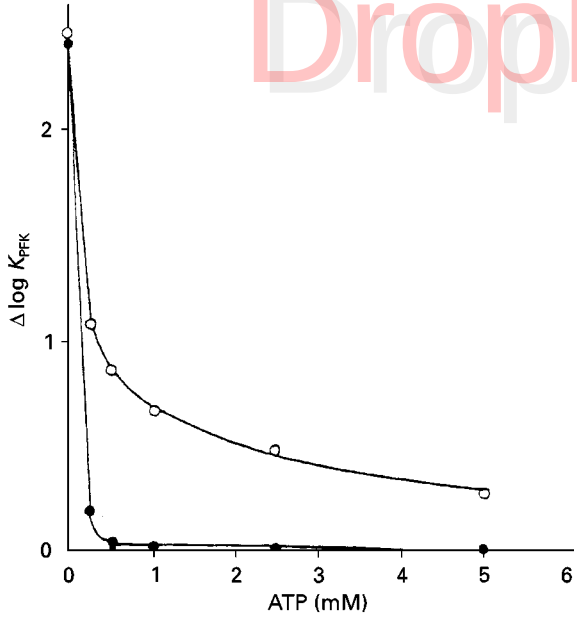


Figure 6 The effect of adenosine triphosphase (ATP) and of ATP + Mg^{2+} on the partitioning of phosphofructokinase from bakers' yeast in a system containing Cibacron blue F3G-A PEG (Cb–PEG). Δ log K of enzyme as function of concentration of ATP. Without addition of Mg^{2+} (○); and with 10 mM $MgCl_2$ (●). System composition: 7%w/w dextran 500 and 5% w/w PEG 8000 including 0.5% Cb–PEG (of total PEG). 50 mM sodium phosphate buffer pH 7.0, 0.5 mM EDTA, 5 mM 2-mercaptoethanol, and 4 nkat g^{-1} enzyme. Temperature, 0°C.

Types of Affinity Ligands Used

A number of affinity ligands have been used and some are presented in **Table 2**. The attachment of ligand to polymers and the purification of the ligand–polymer differs from case to case. Some ligands such as reactive texile dyes can be bound directly to PEG and to dextran in water solution of high pH. Other ligands are introduced by reactions in organic solvent, such as the attachment of acyl groups to PEG by reaction with acyl chloride in toluene. PEG may also be transformed into a more reactive form such as bromo-PEG, tosyl-PEG or tresyl-PEG. Some reaction pathways are shown in **Figure 7**. A number

Table 2 Examples of affinity partitioning

Partitioned substance	*Ligand*
Colipase	Lecithin
Dehydrogenases and kinase	Textile dyes
α-Fetoprotein	Remazol yellow
Haemoglobin and phosphovitin	Cu(II)-chelate
Liver plasma membranes	Lectin
Myeloma protein	Dinitrophenol
Nucleic acids	Dyes
Oxosteroid isomerase	Oestradiol
Red blood cells	Antibodies
Serum albumins, histones and lactalbumin	Fatty acids
Synaptic membranes	Opiates and antagonists
Trypsin	*p*-Aminobenzamidine

DropBooks

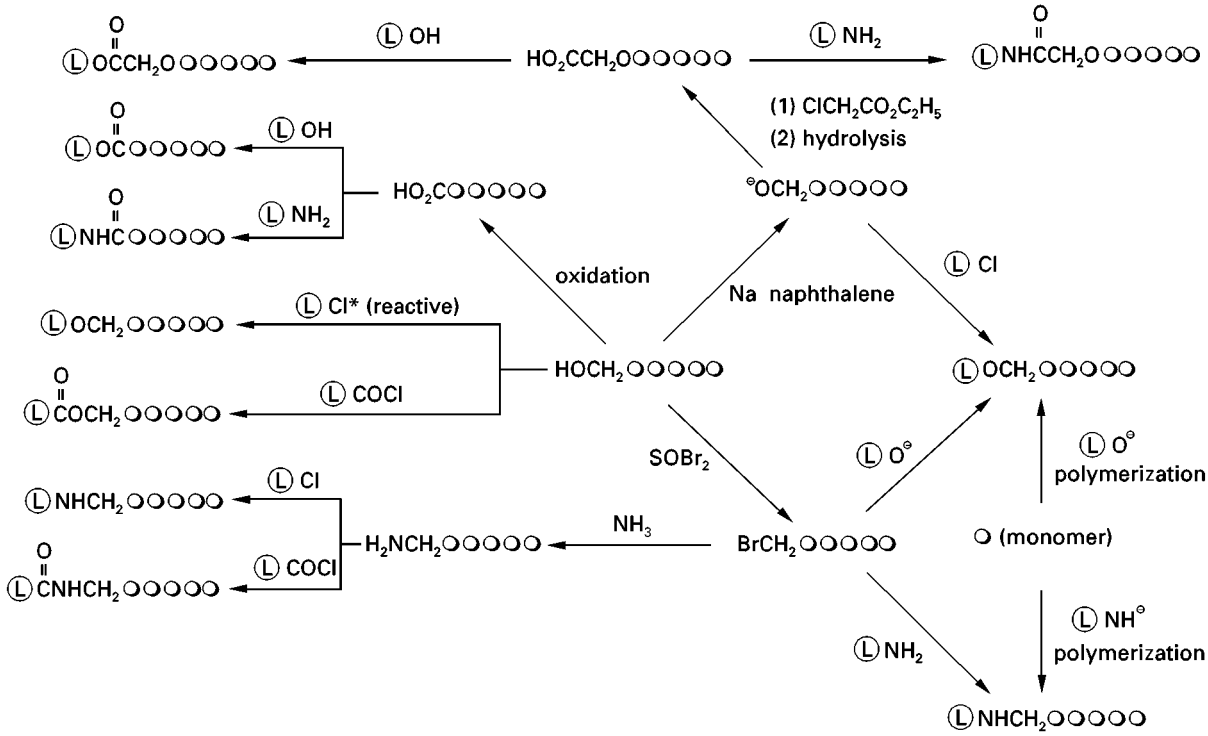


Figure 7 Some reactions used for the covalent linkage of ligands to polymers, preferentially to PEG. The encircled 'L' represents the ligand and the open circles the polymer chain.

of methods to synthesize polymer derivatives have been published by Harris.

Preparative Extractions

The following steps may be useful for a high degree of purification by affinity partitioning.

1. Pre-extraction in a system without ligand–PEG to remove proteins with relatively high partition coefficients. The target protein stays in the bottom phase by adjusting the choice of polymer concentration, salt and pH.
2. Affinity partitioning is carried out by changing the top phase for one containing ligand–PEG. The target protein will now be in the top phase.
3. Washing the top phase with bottom phase to remove co-extracted proteins.
4. 'Stripping' of protein from the affinity ligand by addition of highly concentrated phosphate solution (50%w/w) to the separated upper phase. This generates a PEG-salt two-phase system with PEG and ligand–PEG in the top phase and target protein in the salt-rich bottom phase. An alternative stripping procedure can be carried out by adding a new pure dextran phase to the recovered top phase and supplying the system with free ligand. In this case the target protein will be collected in the lower phase.

For each step the number of extractions and the most suitable volume ratios for yield and purity can be optimized. The procedure is summarized in **Figure 8**.

The yield in the top phase, Y_T, can be calculated from the K value of target protein and the volumes of top and bottom phase, V_T and V_R, respectively, using the following equation:

$$Y_T\,(\%) = \frac{100}{1 + V_B/(V_T K)}$$

and the yield in the bottom phase, Y_B

$$Y_B\,(\%) = \frac{100}{1 + V_T K/V_B}$$

A considerable concentration of the target protein, in addition to purification, can be achieved by choosing an extreme volume ratio with a small collecting phase.

An example of preparative extraction of an enzyme by applying the method given in Figure 8 is the purification of lactate dehydrogenase (LDH) using a PEG-bound textile dye. Crude extract of pig muscle,

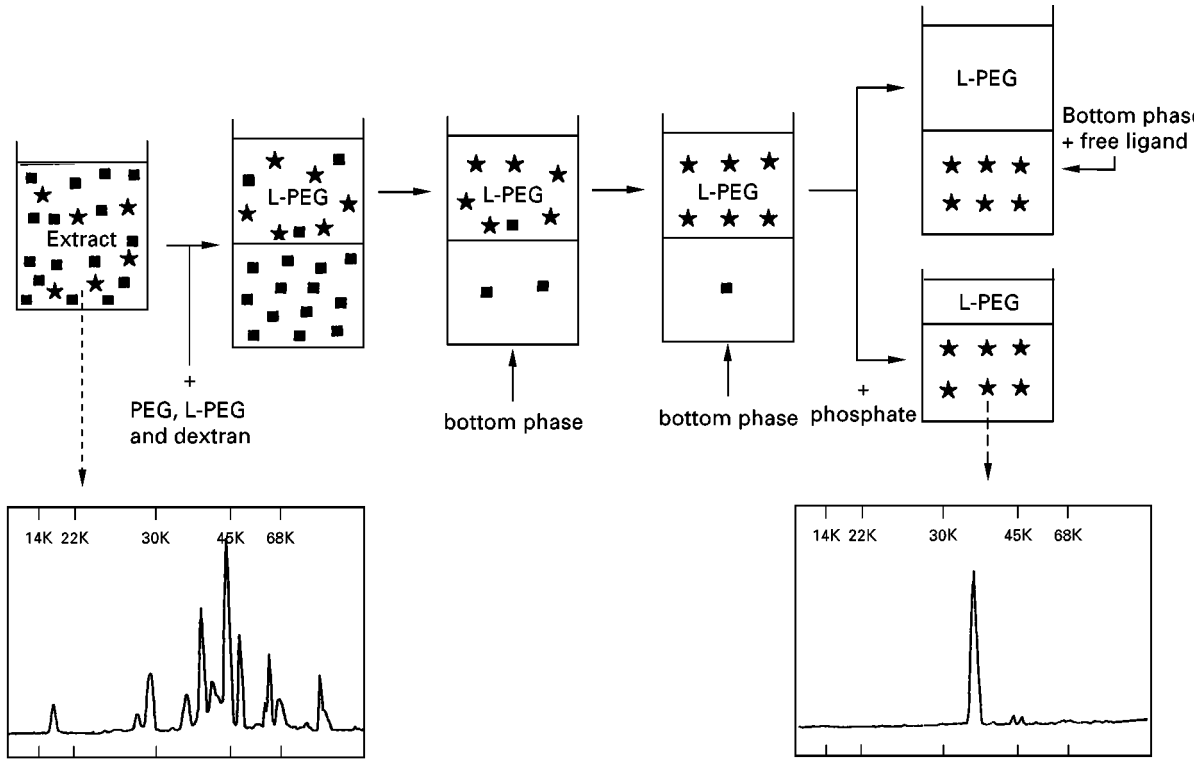


Figure 8 Scheme for the purification of an enzyme (★) from contaminating proteins (■) by using four partitioning steps and PEG–dextran two-phase systems with PEG-bound ligand. This approach has been used for the purification of lactate dehydrogenase (LDH) from meat juice by affinity partitioning with Procion yellow HE-3G PEG. The inserted SDS-PAGE patterns of the original meat extract and the final product (obtained in the phosphate-rich phase) show the removal of contaminating proteins. Recovery of enzyme = 79%. System composition: 10%w/w dextran 500 and 7.1%w/w PEG 8000 including 1% Procion yellow HE-3G PEG (of total PEG), 50 mM sodium phosphate buffer pH 7.9, and 25% w/w muscle extract. Temperature, 0°C. (Reprinted from Johansson G and Joelsson M (1986) *Applied Biochemistry Biotechnology* 13: 15–27, with permission from Elsevier Science.)

cleared by centrifugation, is mixed with PEG, dextran and Procion yellow HE-3G PEG. After the first partitioning the top phase is washed twice with pure lower phases and then it is mixed with a 50% w/w salt solution (25% NaH_2PO_4 + 25% $Na_2HPO_4 \cdot H_2O$). The protein content of the final product in the salt-rich phase compared with that of the initial extract is demonstrated by the polypeptide pattern in sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE) shown in Figure 8. The L–PEG (and PEG) recovered in the final top phase is $\geqslant 95\%$ of the initially introduced amount.

Purification of PFK in combination with a precipitation step with PEG before the affinity partitioning step greatly reduces the original volume of enzyme solution. The extraction included both preextraction and washing steps. The final polishing of the enzyme was made by ion exchanger and desalting with gel chromatography. The results can be seen in **Table 3**.

Table 3 Purification of phosphofructokinase from 1 kg (wet weight) bakers' yeast

Purification step	*Volume (ml)*	*Total activity (U)*	*Total protein (mg)*	*Specific activity (U/mg)*	*Purification factor*	*Yield (%)*	*Proteolytic activity[a] (%)*
Homogenate	1370	5400	13 170	0.41	1	100	100
Fractional precipitation with PEG	120	4810	1836	2.62	6.4	89	18
Affinity partitioning	120	3610	153	23.6	58	67	0.9
DEAE–cellulose treatment	40	2520	63	40	98	47	0.4
Gel filtration	4	1625	28	58	142	30	0.05

[a]In the presence of the protease inhibitor phenylmethylsulfonyl fluoride.

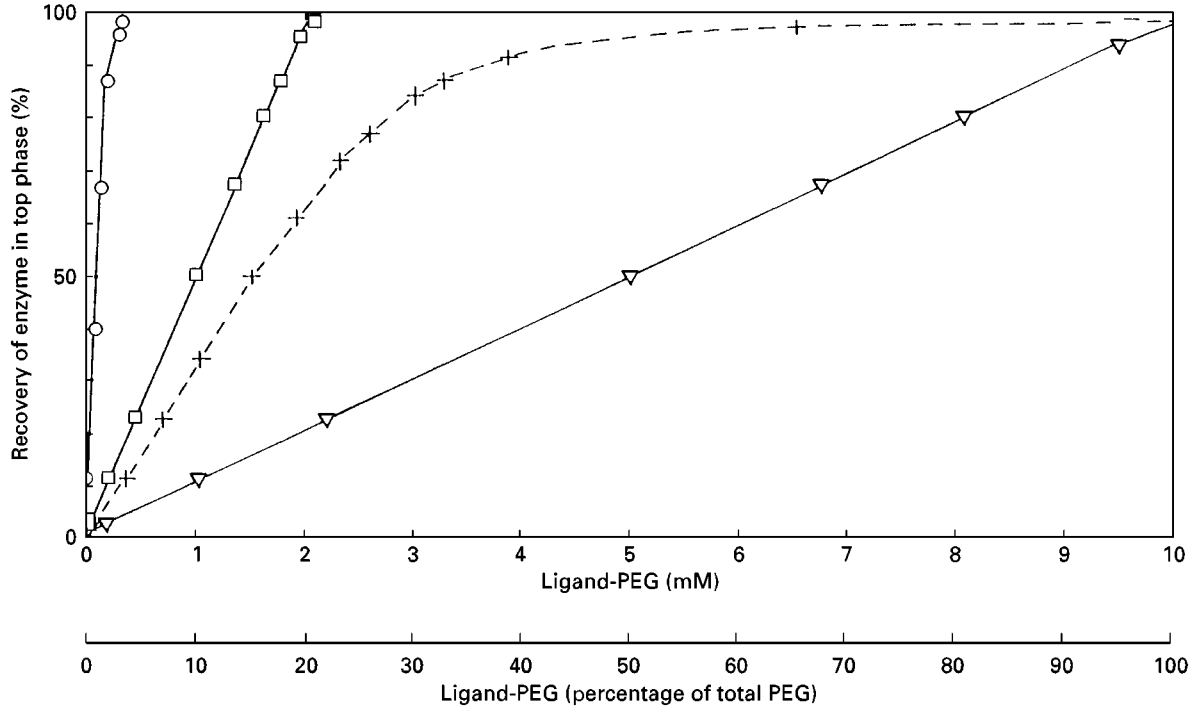

Recovery of enzyme in top phase (%)
100
50
0
0 1 2 3 4 5 6 7 8 9 10
Ligand-PEG (mM)
0 10 20 30 40 50 60 70 80 90 100
Ligand-PEG (percentage of total PEG)

Figure 9 Affinity extraction into the top phase, by using increasing amount of PEG-bound ligand, calculated for an enzyme with the mole mass 100 000 g mol $^{-1}$, containing two binding sites for the ligand, and with $K_P = 0.01$. The value for the partition coefficient, K_L, of the ligand is 100. The association constant, K, for each site is 10^{-6} M^{-1} (○, □) or 10^{-4} M^{-1} (+). The concentration of enzyme: 10 (○), 100 (□, +) and 500 g L^{-1} (△).

The effectiveness of affinity partitioning depends on the binding strength between ligand and protein. Good extraction is obtained with association constants of 10^4 M^{-1} or more (**Figure 9**). The capacity, based on the amount of ligand in the system, is in the range of several hundred grams of protein per kilogram of system. Affinity extractions with 150 g of protein per kilogram of system have been carried out, and in these cases the two-phase systems strongly change the phase volume ratio while the bulk protein acts as a phase-forming component. In systems with high protein concentration the amount of dextran can be reduced or even excluded.

Countercurrent Distribution

A convenient way of multiextraction is countercurrent distribution (CCD). Here a number of top phases are sequentially moved over a set of bottom phases and equilibration takes place after each transfer. The process can be seen as a step-wise chromatography. The original two-phase system, number 0, contains the sample and after that a number (n) of transfers have been carried out $n + 1$ systems are obtained and the various proteins in the sample are distributed along the CCD train. The CCD process is visualized in **Figure 10**(**A**).

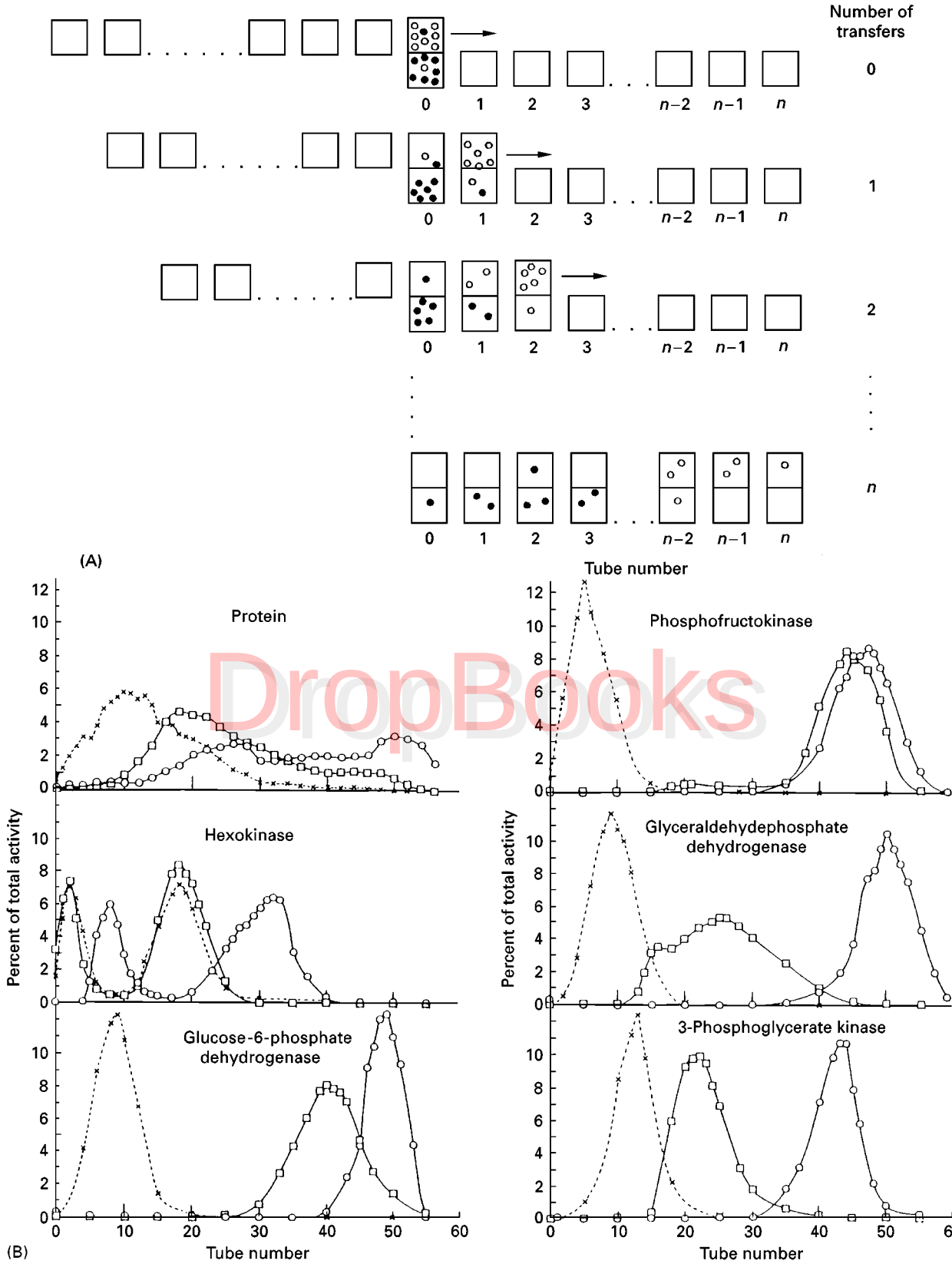
The distribution of a pure substance can be calculated from the K value of the substance and the volumes of the phases, V_T and V_B. Assuming that all of the top phase volume is mobile and all bottom phase stationary, the fractional amount, $T_{n,i}$, in tube number i (i goes from 0 to n) after n transfers will be given by:

$$T_{n,i} = \frac{n!}{i!\,(n-i)!}\,\frac{G^i}{(1+G)^n}$$

This makes it possible to calculate the theoretical curve for a substance and to make comparisons with the experimental distribution curve. Such an analysis may reveal the presence of several components even if they are not separated into discrete peaks. Figure 10(B) shows an example of a CCD of a yeast extract using PEG-bound affinity ligands. The distribution of a number of enzyme activities has been traced.

Use of Dextran as a Ligand Carrier

Dextrans of the molecular weights normally used (40 000 and 500 000 Da) contain many thousands of reactive hydroxyl groups per molecule. The affinity partitioning effect achieved by introducing



DropBooks

Figure 10 (A) Scheme of the countercurrent distribution (CCD) process. (Reprinted from Johansson G, Andersson M and Akevland HE (1984) *Journal of Chromatography* 298: 485–495. With permission from Elsevier Science.) (B) Distribution of protein and some glycolytic enzymes after CCD of an extract of bakers' yeast using 55 transfers. Without ligand-PEG (×); with Procion Olive MX-3G PEG, 1% of total PEG (□); and with Procion yellow HE-3G PEG, 1% of total PEG (○). System composition: 7% w/w dextran 500 and 5%w/w PEG 8000 including ligand–PEG, 50 mM sodium phosphate buffer pH 7.0, 0.2 mM EDTA, and 5 mM 2-mercaptoethanol. Temperature, 3°C. Systems in chamber 0–2 were initially loaded with yeast extract.

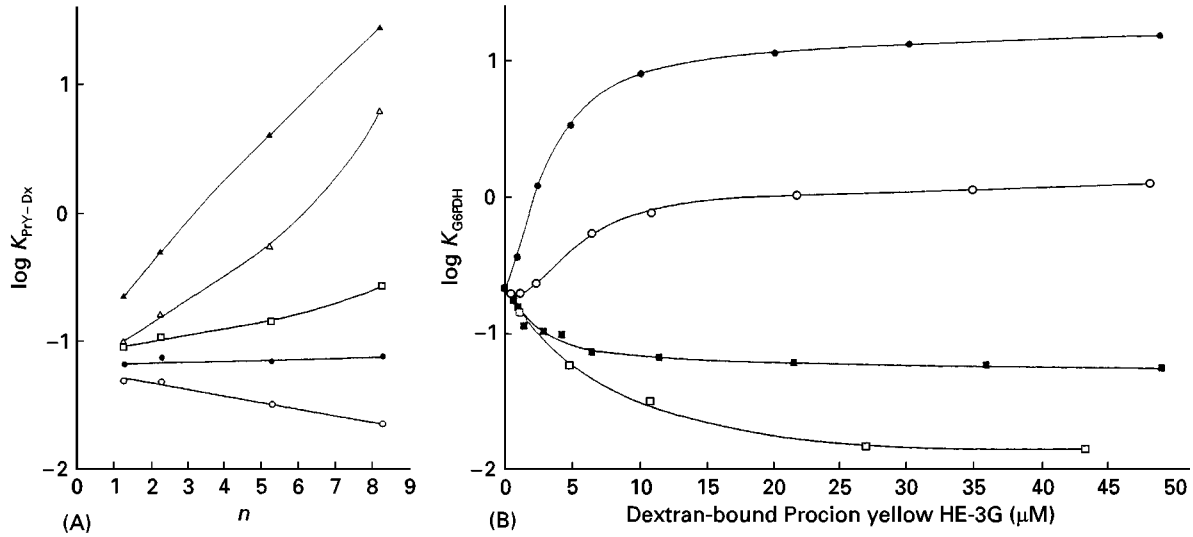


Figure 11 (A) Partitioning of Procion yellow HE-3G dextran 70 (PrY–Dx) depending on the degree of substitution, *n* (expressed in molecules of dye bound per molecule of dextran), in systems containing 50 mM sodium phosphate buffer (▲); 10 mM sodium sulfate (△); 100 mM sodium acetate (□); 100 mM KCl and 5 mM sodium phosphate buffer (●); or 100 mM $KClO_4$ (○), at pH 7.9. Arrow indicates *K* of unsubstituted dextran. System composition: 8%w/w dextran 70 and 4.5%w/w PEG 8000 including PrY–Dx (50 μM bound dye), and indicated salt. Temperature, 22°C and pH of system adjusted to 7.9. (Reprinted from Johansson G and Joelsson M (1987) *Journal of Chromatography* 411: 161–166. With permission from Elsevier Science.) (B) Effect of the concentration of PrY–Dx on the partitioning of the enzyme glucose-6-phosphate dehydrogenase (G6PDH) using PrY–Dx with *n* equal to 1.3, □; 2.3, ■; 5.3, ○; and 8.3, ●. System as in (A) with 50 mM sodium phosphate buffer.

one or just a few dye ligands is shown in **Figure 11**. Since the dye ligands used here carries seven to ten charged groups per molecule they also add a considerable (negative) net charge to the ligand dextran. Its partitioning will then be sensitive to the presence of salt and the choice of salt. The ligand–dextran can be directed either to the bottom phase or the top phase. This steering is more effective the greater the number of ligands per dextran molecule.

The effect of ligand–dextran on the partitioning of an enzyme, glucose-6-phosphate dehydrogenase, is shown in Figure 11. There is also a tendency towards affinity precipitation when the concentration of ligand molecules is equal to the concentration of enzyme binding sites in the system. This is seen as a shallow dip in the extraction curve.

Use of a Third Polymer as Ligand Carrier

The ligand can be bound to a third polymer chosen in such a way that it will be mainly concentrated in one phase. Alternatively, if it is carrying enough charged groups, it may be steered to one phase by using salts. The efficiency, measured as $\Delta \log K / \log K_L$, equal to the apparent number of binding sites of the protein, has in several cases showed that the most effective polymer for carrying the ligand is neither of the two phase-forming polymers. The effect of a dye ligand, bound to various polymers, on the partitioning of lactate dehydrogenase in a dextran–PEG system is presented in **Table 4**.

Table 4 The effect of ligand carrier on its efficiency in producing affinity partitioning

Ligand carrying polymer	$\Delta \log K_{LDH}$	$\log K_{L\ -polymer}$	$n_{app} = \Delta \log K_{LDH} / \log K_{L\ -polymer}$
Ficoll	2.11	0.90	2.3
Hydroxypropylstarch	0.59	0.32	1.8
Poly(ethylene glycol)	2.23	1.50	1.5
Dextran (D.S. = 8.3)	2.31	1.60	1.4
Ethylhydroxyethyl cellulose	2.06	1.63	1.3

Lactate dehydrogenase (LDH) was partitioned in systems containing 7% (w/w) dextran 500, 5% w/w PEG 8000, 25 mM sodium phosphate buffer, pH 7.5, and Procion yellow HE-3G polymer of dye concentration of 42 μM. Temperature, 22°C. (Reprinted from Johansson and Joelsson M (1984) *Journal of Chromatography* 411: 161–166. With permission from Elsevier Science.)

Chiral Affinity Partitioning

For separation of low molecular weight substances into their enantiomeric forms a system may be used where one of the phases contains a high molecular weight substance which binds one of the enantiomers. Bovine serum albumin as well as cyclodextrin have been used for this purpose.

Analytical Uses

Besides the preparative use of aqueous two-phase systems, they have been applied to a number of analytical studies of the properties of biological macromolecules and particles. Some of these uses are binding studies, conformational changes, studies of antibodies, and homogeneity studies of protein, nucleic acids, membranes, organelles and cells.

Multiphase Systems

By using more than two polymers, multiphase systems can be obtained. In principle, the number of phases can be as many as the kinds of polymers used. A three-phase system of PEG, Ficoll, and dextran has been used with two ligands (in different phases) for directing the partitioning of blood serum proteins.

Semi-organic Systems

Part of the water in a two-phase system may be replaced by certain solvents. Often dextran cannot be used because of low solubility but it may be replaced by Ficoll. The log K of a protein may change drastically by introducing the organic solvent. Also the $\Delta \log K$ may in some cases be reduced while it in other cases has been found to remain relatively uneffected.

Affinity Partitioning of Nucleic Acids and Bioparticles

Affinity partitioning in aqueous two-phase systems is not restricted to proteins, but has been also used for purification of DNA, using base-pair specific ligands, membrane fragments, and cells, such as erythrocytes. Some examples of such affinity extractions are found in Table 2.

Future Prospects

More specific ligands will certainly come into use for affinity partitioning and systems with much larger partition coefficients will be developed. This will allow not only specific extraction of biomaterials but also their many-fold concentration. Effective recycling processes of ligand–polymers will make it economically feasible to use affinity partitioning for extraction of enzymes on a technical scale. Successive extraction of several components from one and the same source by using a number of ligands in series extraction can be foreseen.

Conclusions

Affinity partitioning is a method of selective liquid–liquid extraction for purification and studies of proteins and other 'water stable' cell constituents. The scaling up of this process is uncomplicated and the recovery of ligand polymer reduces the cost.

See also: **I/Affinity Separation. II/Affinity Separation:** Dye Ligands; Rational Design, Synthesis and Evaluation: Affinity Ligands. **III/Nucleic Acids:** Extraction. **Proteins:** Electrophoresis; High-Speed Countercurrent Chromatography; Ion Exchange. **Appendix 1/Essential Guides for Isolation/Purification of Cells. Essential Guides for Isolation/Purification of Enzymes and Proteins. Essential Guides for Isolation/Purification of Nucleic Acids.**

DropBooks

Further Reading

Albertsson PA (1986) *Partition of Cell Particles and Macromolecules*, 3rd edn, pp. 334–340. New York: John Wiley.

Albertsson PA and Birkenmeier G (1988) Affinity separation of proteins in aqueous three-phase systems. *Analytical Biochemistry* 175: 154–161.

Flanagan SD and Barondes SH (1975) Affinity partitioning – a method for purification of proteins using specific polymer-ligands in aqueous polymer two-phase systems. *Journal of Biological Chemistry* 250: 1484–1489.

Harris JM (1985) Laboratory synthesis of polyethylene glycol derivatives. *Journal of Macromolecular Science. Reviews of Polymer Chemistry and Physics*. C25: 325–373.

Johansson G (1995) Multistage countercurrent distribution. In: Townshend A (ed.) *The Encyclopedia of Analytical Science*, pp. 4709–4716. London: Academic Press.

Johansson G and Joelsson M (1987) Affinity partitioning of enzymes using dextran-bound Procion yellow HE-3G. Influence of dye-ligand density. *Journal of Chromatography* 393: 195–208.

Johansson G, Kopperschläger G and Albertsson PA (1983) Affinity partitioning of phosphofructokinase from baker's yeast using polymer-bound Cibacron blue F3G-A. *European Journal of Biochemistry* 131: 589–594.

Kopperschläger G and Birkenmeier G (1990) Affinity partitioning and extraction of proteins. *Bioseparation* 1: 235–254.

Tjerneld F, Johansson G and Joelsson M (1987) Affinity liquid–liquid extraction of lactate dehydrogenase on a large scale. *Biotechnology and Bioengineering* 30: 809–816.

Walter H and Johansson G (eds) (1974) *Methods in Enzymology*, Vol. 228, *Aqueous Two-phase Systems*. San Diego, CA: Academic Press.

Aqueous Two-Phase Systems

See **II/AFFINITY SEPARATION/Affinity Partitioning in Aqueous Two-Phase Systems**

Biochemical Engineering Aspects of Affinity Separations

H. A. Chase, University of Cambridge, Cambridge, UK

Copyright © 2000 Academic Press

Introduction

Affinity separations are popular methods for the purification of biological molecules and other biological entities. They can readily be implemented on the laboratory scale but a number of additional factors have to be considered when these techniques are to be used for production purposes. Under these circumstances it is necessary to apply biochemical engineering principles to the design, scale-up and optimization of affinity separations. These topics are the subject of this article.

Selective interactions are exploited in affinity separations in order to achieve greater adsorbent selectivity for the desired molecule. Subtle differences in physical properties such as charge, size and hydrophobicity are often found to be insufficient for the required degree of purification in many separations of biological compounds. Many separations require the isolation of a minority component from a highly complex feedstock which may contain large amounts of similar compounds. As a consequence, it has been necessary to devise recovery flow sheets that consist of an extensive sequence of different steps – a sequence that may result in low overall yields and excessive costs. Hence affinity separations have been developed as alternatives to the more widely used separations based on ion exchange, hydrophobic interaction and size exclusion methods. Provided a ligand can be obtained which is truly selective for the desired component, it is possible to recover that component from a complex feedstock to a high degree of purity and in high yield. Typically the ligand is used in heterogeneous phase separations in which it is immobilized on to the surfaces of a porous solid-phase matrix material and employed in chromatographic and other adsorption techniques. Other approaches including the use of affinity ligands in selective precipitation and in modifying the phase selectivities in aqueous two-phase separations (ATPS) have been reported, but are not considered further here.

A variety of ligands with a wide range of molecular complexities have been developed for use in affinity separations and these are reviewed extensively elsewhere in this work. In many examples, duplication of the selective interactions that occur during the normal function of biomolecules have been exploited during such affinity separations; the affinity ligand is frequently one of the components of a recognition interaction. Examples include the recognition between an enzyme and its inhibitor or co-factor, or the highly specific interaction between an antigen and an antibody raised against it. Biomimetic molecules have been developed to mimic the recognition sites of more complex molecules, either by exploiting fortuitous interactions shown by readily available compounds (e.g. textile dyes) or as a result of the identification of new compounds either by studying the detailed three-dimensional structure of the target, or by the techniques of combinatorial synthesis. Selective molecular recognition can also be achieved without mimicking any naturally occurring